--100. (New) The composition of claim 57, wherein the conjugation of the ganglioside involves a ceramide double bond of the ganglioside and a reactive amine group of the Keyhole Limpet Hemocyanin or a derivative thereof.--

F4 cont

--101. (New) The composition of claim 57, wherein the conjugation of the ganglioside involves a ceramide double bond of the ganglioside and an aminolysyl group of the Keyhole Limpet Hemocyanin or a derivative thereof.--

REMARKS

Claims 57-77 were pending in the subject application. Applicants have hereinabove amended claims 57, 62-64, 71-72 and added new claims 78-101. Support for these amendments may be found inter alia in the specification on page 12, lines 4-14, page 32, line 1 to page 33, line 10, page 76, lines 19-21 and page 114, line 20 to page 116, line 14. Applicants contend that this amendment does not involve any issue of new matter. Entry of this amendment is respectfully requested such that claims 57-101 will be pending.

Status of Claims

Applicants acknowledge the Examiner's statement that the amendment filed October 1, 1998 has been entered into the record and that claims 57-77 are now pending.

Claims 57-70

The Examiner stated that newly submitted composition claims 57-70 are directed to an invention that is independent or distinct from the invention originally claimed for the following reasons: the compositions as claimed are distinct because they can be used in a materially different process such as linked to a column for purification of cross reactive antibodies, in an in vitro method to

study immune responses or in an in vitro method to generate monoclonal antibodies.

The Examiner stated that since applicant has received an action on the merits for the originally presented methods invention, this invention has been constructively elected by original presentation for prosecution on the merits. The Examiner stated that accordingly, claims 57-70 are withdrawn from consideration as being directed to a non-elected invention. The Examiner stated to see 37 CFR 1.142(b) and MPEP § 821.03.

The Examiner stated that this application contains claims 57-70 drawn to an invention nonelected by original presentation. The Examiner stated that a complete reply to the final rejection must include cancellation of nonelected claims or other appropriate action (37 CFR 1.144) See MPEP § 821.01.

In response, applicants respectfully traverse the Examiner's above objection. Nevertheless, without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application, applicants have hereinabove amended claims 57, 62-64, and 71-72 and added new claims 78-101. Furthermore, applicants note that 35 U.S.C. §121 states, in part, that "[i]f two or more independent and distinct inventions are claimed in one application, the Commissioner may require the application to be restricted to one of the inventions." [Emphasis added].

Applicants request that the Examiner withdraw her withdrawal from consideration of claims 57-70 as being directed to a non-elected invention in view of the fact that claims 57-70 are not independent of claim 71-77. Applicant maintains that these claims do not define patentably distinct inventions.

Under M.P.E.P. §802.1, "independent" means "there is no disclosed relationship between the subjects disclosed, that is, they are unconnected in design, operation, and effect." Claims of 57-70 drawn to a composition comprising a ganglioside conjugated through a ceramide-derived carbon of the ganglioside to Keyhole Limpet Hemocyanin or a derivative thereof and an adjuvant, the amounts of such conjugated ganglioside and such adjuvant being effective to stimulate or enhance antibody production in a subject, and a pharmaceutically acceptable carrier, wherein the Keyhole Limpet Hemocyanin derivative comprises Keyhole Limpet Hemocyanin linked to an immunological adjuvant. These claims are related to claims 71-77 which are drawn to methods of use of this composition for stimulating or enhancing antibody production and of preventing or treating a cancer. Accordingly, all of the claims are related to the conjugation of a ganglioside through a ceramide-derived carbon of the ganglioside to Keyhole Limpet Hemocyanin or a derivative thereof effective to stimulate or enhance antibody production in a subject. Therefore, these claims are related.

Applicants therefore respectfully assert that two or more independent and distinct inventions have not been claimed in the subject application because these claims are not independent under M.P.E.P. §802.01. Therefore, these claims should all be examined.

Additionally, Applicant points out that under M.P.E.P. §803, the Examiner must examine the application on the merits, even though it includes claims to distinct inventions, if the search and examination of an application can be made without serious burden. There are two criteria for a proper requirement for restriction, namely (1) the invention must be independent and distinct; AND (2) there must be a serious burden on the Examiner if restriction is not required.

Applicant maintains that there would not be a serious burden on the Examiner to examine all of the claims. A search of prior art with regard to claims 71-77 will reveal whether any prior art exists as to claims 57-70. Since there is no burden on the Examiner to examine these claims in the subject application, the Examiner must examine the entire application on the merits.

Applicant maintains that claims 57-101 define a single inventive concept. Accordingly, Applicant respectfully requests that the Examiner reconsider and withdraw this ground of objection and examine claims 57-101 on the merits.

Obviousness-type Double Patenting Rejection

The Examiner provisionally rejected claims 71-77 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 65-71 of copending Application No. 08/477,097. The Examiner stated that although the conflicting claims are not identical, they are not patentably distinct from each other because they all claim conjugating proteins to gangliosides through the ceramide portion and thus the particular method species drawn to GM2 or GM3 claimed in the copending application would anticipate the instant genus method claims.

The Examiner provisionally rejected claims 71-77 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 66-72 of copending Application No. 08/475,784. The Examiner state that although the conflicting claims are not identical, they are not patentably distinct from each other because they all claim conjugating proteins to gangliosides through the ceramide portion and thus the particular method species claimed in the copending application would anticipate the instant genus method claims.

The Examiner provisionally rejected claims 71-77 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 66-72 of copending Application No. 08/196,154. The Examiner state that although the conflicting claims are not identical, they are not patentably distinct from each other because they all claim conjugating proteins to gangliosides through the ceramide portion and thus the particular method species claimed in the copending application would anticipate the instant genus method claims.

In response, applicants respectfully traverse the Examiner's above provisional obviousness-type double patenting rejections becsue applicants have hereinabove amended the claims. Furthermore, applicants respectfully point out that for a provisional double patenting rejection, M.P.E.P. §804 requires that the

> 'provisional' double patenting rejection should continue to be made by the Examiner in each application as long as there are conflicting claims in more than one application unless that 'provisional' double patenting rejection is the only rejection remaining in one of the applications. If the 'provisional' double patenting rejection in one application is the only rejection remaining in that application, the Examiner should then withdraw that rejection and permit the application to issue as a patent, thereby converting the 'provisional' double patenting rejection in the application(s) into a double patenting rejection at the time the one application issues as a patent.

Since none of the above-mentioned copending applications have yet issued as a patent, applicants maintain that even if the Examiner continues to conclude that the claims of the subject application

conflict with the claims of the copending applications, the provisional rejection should be withdrawn in view of applicants' arguments which overcome the other rejections of this application. Applicants contend that these remarks obviate the above rejections and respectfully request that the Examiner reconsider and withdraw the rejections.

Rejection under 35 U.S.C. 112, first paragraph

The Examiner rejected claims 71-77 under 35 U.S.C. 112, first paragraph for reasons made of record for claims 44 and 46-56 in Paper No. 13, mailed 4-1-98.

The Examiner stated that applicants' have asserted that the claims are no longer drawn to vaccines and thus the issues with regard to effective treatment or prevention of cancer of record in the last office action is moot. The Examiner stated that this is not persuasive because the claims 72-77 are clearly drawn to treatment and prevention of cancer by administration of an agent which the specification teaches generates an antibody response. The Examiner stated that the applicants have not provided any evidence the administration of the composition whether or not entitled vaccine has any effect for treatment. The Examiner stated that applicants have not provided any evidence to show that the administered agents prevent cancer in any disease state. The Examiner stated that the claims remain not enabled for reasons already made of record. The Examiner stated that the compositions are taught in the specification to act through enhancement of the immune response and are thus still considered "vaccines" in as much as, vaccines encompass any prophylactic or therapeutic material containing antigens on administration to man will stimulate active immunity (see The Dictionary of Immunology attached) and it is this active immunity which applicants propose throughout the specification

which is allegedly capable of treating or preventing cancer. The Examiner stated that thus, claims drawn to treatment and prevention of cancer are not enable for reasons already made of record.

In response, applicants respectfully traverse the Examienr's above rejection. In support of claims concerning the treatment and/or prevention of the cancer, applicants attach hereto as **Exhibit B** a copy of the following paper: Helen Zhang et al "Antibodies against GD2 Ganglioside Can Eradicate Syngeneic Cancer," Cancer Research 58: 2844-2849 (1998). This paper demonstrates that the conjugated vaccine of the subject invention prevents the outgrowth of micrometastases (see page 2844, first column). The paper shows that the conjugated vaccine prevents establishment of subsequently administered EL4 challenge (which is a lymphoma), and eliminates EL4 micrometastases when administered after EL4 challenge (see page 2844, second column). The paper shows that mice receiving the conjugate vaccine survived significantly longer, and that one mouse did not show any evidence of tumor (see page 2845, second column). The paper teaches that the conjugated vaccine protects against tumor challenge and eliminates micrometastases. Applicants contend that this is support for the use of applicants claimed invention to treat or prevent cancer and also to prevent the relapse of a cancer. Applicants contend that these remarks obviate the above rejections and respectfully request that the Examiner reconsider and withdraw the rejection.

Derivative

The Examiner stated that the applicants' arguments regarding the issue of "derivative" have been carefully considered but are not persuasive for the reasons set forth below. The Examiner stated that applicants' argue that derivatives have been taught and point to page 12, lines 4-13. The Examiner stated that this passage is

not persuasive because the derivatives are in fact conjugates with other proteins and is thus not commensurate in scope with the claims. The Examiner stated that the term derivative clearly encompasses changes to the primary structure of Keyhole Limpet Hemocyanin (KLH). The Examiner stated that derivative is not defined in the specification or the claims to exclude amino acid changes and modifications of the primary amino acid structure of KLH. The Examiner stated that the derivatives disclosed by applicants are n fact conjugates with other proteins and thus applicants arguments are not commensurate in scope. The Examiner stated that applicants' provide no guidance as to which changes in KLH residues or derivations of amino acids would function appropriately and thus the skilled artisan would have to resort to unguided experimentation. The Examiner stated that absent a guidance for modification of the sequence per se, any experimentation would be undue. The Examiner stated that the specification fails to provide even the most rudimentary guidance for a starting point for chemical derivation of the KLH sequence other than conjugation with other proteins. The Examiner stated that thus, applicants arguments are not commensurate in scope with the scope of "derivative". The Examiner stated that the rejection is maintained for reasons made of record.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants contend that the claimed invention was fully enabled and that undue experimentation would not be needed to practice the invention. Nevertheless, without conceding the correctness of the Examiner's position but to expedite the prosecution of the subject application, applicants have hereinabove amended and added new claims such that the derivative thereof comprises KLH linked to an immunological adjuvant, and furthermore added claims such that the immunological adjuvant is a

monophospholipid A, a non-ionic block copolymer or a cytokine. Applicants contend that the specification fully supports claims drawn to linking of KLH to an immunological adjuvant. Applicants respectfully point the Examiner's attention to page 12, lines 4-14 for such support. Applicants contend that the claims that will be pending upon the entry of this amendment are fully enabled and that one skilled in the art would be able to practice the invention without undue experimentation. Applicants contend that these remarks and amendments obviate the above rejections and respectfully request that the Examiner reconsider and withdraw the rejection.

Rejection under 35 U.S.C. 112, second paragraph

The Examiner rejected claims 72-77 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner stated that the claims are rendered indefinite because they depend from claims withdrawn from consideration as drawn to an invention non-elected by original presentation. The Examiner stated that correction is required.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants contend, for the reasons stated supra pages ___ that claims 72-77 are not indefinite as being drawn to a non-elected invention. Applicants respectfully request that the claims 57-101 be examined on the merits such that claims 72-77 are not drawn to a nonelected invention. Applicants contend that these remarks obviate the above rejection and respectfully request that the Examiner reconsider and withdraw the rejection.

Rejection Under 35 U.S.C. 112, first paragraph

The Examiner rejected claims 71-77 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner stated this is a new matter rejection.

The Examiner stated that claims now recite the phrase "..comprising a ganglioside conjugated through the ceramide portion of the ganglioside to a Keyhole Limpet Hemocyanin..". The Examiner stated that this phrase and the concept of this type of conjugation is not supported by the written description of the specification as originally filed. The Examiner stated that the passages to which applicants' point for support, fail to convey conjugation of the ganglioside through the ceramide portion. The Examiner stated that these passages do not even mention the ceramide portion. The Examiner stated that this issue is best resolved by applicants pointing to the specification by page and line number where support for the now claimed limitation is found.

In response, applicants respectfully traverse the Examiners above rejection. Applicants contend that the claimed subject matter was sufficiently described in the subject application to reasonably convey to one skilled in the art that applicants had possession of the claimed invention at the time of filing. In support, applicants respectfully direct the Examiner's attention to page 32, line 1 to page 33, line 10 of the specification. Contrary to the Examiner's statement, the concept of the ceramide portion is specifically discussed on page 32, lines 13-18.

Rejection under 35 U.S.C. 103(a)

The Examiner rejected claims 71-77 under 35 U.S.C. 103(a) as being

unpatentable over Livingston et al (Cancer Research, 49:7045-7050, 1989) in view of Ire et al (U.S. Patent No. 4,557.931, published December 10, 1985) and Ritter et al (Cancer Biology, 2:401-409, 1991).

The Examiner stated that Livingston et al (Cancer Research, 49:7045-7050, 1989) teach a composition administered to melanoma patients for stimulating the production of antibodies directed to a carbohydrate epitope on the ganglioside, GM2 (p7046-7048). The Examiner stated that Livingston et al teach that the Gm2 is administered in conjunction with an adjuvant, Bacillus Calmette-Geurin (BCG), and a pharmaceutically acceptable vehicle, phosphate buffered saline (p 7048, column 1, paragraph 3 and paragraph bridging p 7046-47). The Examiner stated that Livingston et al teach that the melanoma recurrence was delayed in patients developing GM2 antibodies after vaccination (p 7048- pargaraph 1, and column 2, paragraph 2). The Examiner stated that Livingston et al teach that more patents produced IgM antibodies that IgG antibodies to the GM2)p 7047 paragraph bridging column 1-2). The Examiner stated that Livingston et al also teach the gangliosides GM2, GD2, and GD3 are expressed on the cell surface of human malignant melanomas (p 7045, column 1, paragraph 2). The Examiner stated that Livingston et al don not teach the conjugation of the GM2 vaccine with Keyhole Limpet Hemocyanin (KLH) through the ceramide portion of the ganglioside or use of any of the other gangliosides in a method to induce an immune response or cancer treatment.

The Examiner stated the Irie et al teach conjugation of ganglioside GM2 to a non-toxic protein carrier, such as albumin, using ozonolysis (column 5, see B., lines 19-68) which conjugates a the GM2 through the ceramide portion. The Examiner stated that Irie et al teach that the fatty acid of ceramide maybe removed leaving

sphingosine and thus the coupling takes place through the amine group of the sphingosine moiety (column 2, lines 64-69). The Examiner stated that the Irie et al teach that the conjugated GM2 can be used as a vaccine to stimulate an immune response and raise the anti-GM2 titer in mammals (column 2). The Examiner stated that Irie et al differ by not conjugating the GM2 to KLH.

The Examiner stated that Ritter et al (Cancer Biology, 2:401-409, 1991) teach that the IgG responses to gangliosides may be increased by the covalent attachment of foreign carrier proteins such as KLH to the gangliosides resulting in the T cell help necessary for the response (p 406, paragraph 1). The Examiner stated that Ritter et al discloses the advantage of using an IgG antibody response (versus IgM) against gangliosides is that IgG a) has a higher affinity; b) is better able to penetrate solid tissues; c0 is able to mediated antibody-dependent cell-mediated cytotoxicity; and d0 is generally detectable in the serum for longer periods after immunization.

The Examiner stated that it would have been prima facie obvious to one of ordinary skill in the art to modify the GM2-albumin ceramide conjugate of Irie et al by substituting KLH for albumin and to substitute the resulting GM2-KLH ceramide conjugate for the GM2 in the immunization composition of Livingston et al for active immunization for generating antibody response for melanoma treatment because Irie et al teach that the IgG responses to gangliosides may be increased by the covalent attachment of foreign carrier proteins such as KLH to the gangliosides resulting in the T cell help necessary for the response (p 406, paragraph 1) and Ritter et al discloses the advantages of generating and IgG as opposed to an IgM antibody response and optimization of the dosage, route of administration and number of sites to administration and number of sites to administer the composition as combined above is

well within the skill of the art.

In response, applicants respectfully traverse the Examiner's above rejections. Applicants contend that none of the cited references either alone or in combination teach, disclose or suggest applicants claimed invention. Nevertheless, without conceding the correctness of the Examiner's position but to expedite the prosecution of the subject application, applicants have hereinabove amended the claims. Applicants contend that these amendments obviate the Examiner's above rejections and respectfully request that the Examiner reconsider and withdraw the rejections.

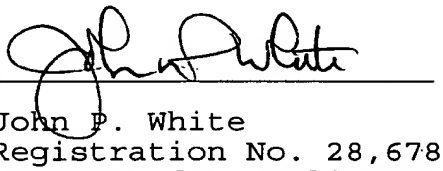
Summary

In view of the foregoing remarks and amendments, applicants respectfully request that the above grounds of rejection and objection be reconsidered and withdrawn and earnestly solicit allowance of the now pending claims.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone at the number provided below.

No fee, other than the enclosed $1516.00 fee which includes the $925.00 fee for a five month extension of time, the $345.00 fee under 37 C.F.R. §1.17(r) and $246.00 fee for additional claims is deemed necessary in connection with the filing of this Communication. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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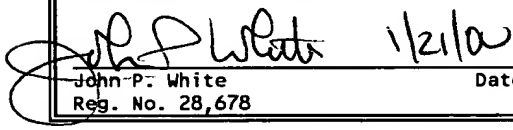
I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.



John P. White Date
Reg. No. 28,678

[CANCER RESEARCH 58, 2844–2849, July 1, 1998]

Antibodies against GD2 Ganglioside Can Eradicate Syngeneic Cancer Micrometastases[1]

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ABSTRACT

After 10 years of clinical trials in patients with advanced cancer, monoclonal antibodies (mAbs) against cell surface antigens have not lived up to their initial promise. One such cell surface antigen is the ganglioside GD2. GD2 is richly expressed at the cell surfaces of human neuroblastomas, sarcomas, and melanomas. We have described a murine lymphoma (EL4) that is syngeneic in C57BL/6 mice and expresses GD2, a mAb against GD2 (mAb 3F8), and we have prepared a conjugate vaccine (GD2-keyhole limpet hemocyanin plus immunological adjuvant QS-21) that consistently induces antibodies against GD2. We demonstrate here, for the first time in a syngeneic murine model, that passively administered and vaccine-induced antiganglioside antibodies prevent outgrowth of micrometastases, and we use this model to establish some of the parameters of this protection. The level of protection was proportional to antibody titer. Treatment regimens resulting in the highest titer antibodies induced the most protection, and protection was demonstrated even when immunization was initiated after tumor challenge. Treatment with 3F8 1, 2, or 4 days after i.v. tumor challenge was highly protective, but waiting until 7 or 10 days after challenge resulted in minimal protection. The results were similar whether number of liver metastases or survival was used as the end point. These results suggest that unmodified mAbs or antibody-inducing vaccines against GD2 (and possibly other cancer cell surface antigens) should be used exclusively in the adjuvant setting, where circulating tumor cells and micrometastases are the primary targets.

INTRODUCTION

Most mAb[3] treatments have been performed on patients with advanced disease, and the treatments were of short duration, with response of measurable disease as the end point. Responses have been rare. Occasional regression of measurable neuroblastoma, melanoma, and breast cancer lesions and more frequent regression of B-cell lymphomas have resulted in patients treated with mAbs against cell surface antigens, including: gangliosides GM2[4] (1), GD2 (2–5), and GD3 (6–8); HER2 neu (9); and lymphoma idiotype antigens (10, 11). Trials with mAbs against GD2 are a case in point. The response rate in children with GD2-positive cancers (primarily neuroblastomas) treated with mAb 14.G2a or 3F8 is between 0 and 25% (12, 13), and in melanoma patients treated with mAb 3F8, 14.G2a, or chimeric 14.18, the response rate is between 0 and 22% (13, 14). A chimeric 14.18-interleukin 2 fusion protein shown to be potent in a *scid/scid* xenograft model (15) is now being considered for clinical trials. Neither immunogenic GD2 vaccines nor a syngeneic animal model has been previously available, making it difficult to compare these various approaches or to test the many variables associated with antibody-mediated therapies in the setting of a normal immune system.

As opposed to the minimal benefit seen with mAbs in patients with advanced disease, there is an expanding body of evidence indicating that antibodies can protect against subsequent tumor challenge in experimental animals and prevent tumor recurrence in humans. mAbs against several protein or glycoprotein tumor antigens have resulted in significant protection from syngeneic tumors in the mouse (16–19), mAb R24 against GD3 has resulted in protection from syngeneic melanoma growth in hamsters (20), and mAbs against GD2/GD3 (21) or GD2 (22) have resulted in protection against human tumor challenges in nude mice. There is also evidence in humans that natural antibodies, passively administered antibodies, or vaccine-induced antibodies against cancer antigens can result in prolonged disease-free and overall survival in the adjuvant setting. (*a*) Paraneoplastic syndromes have been associated with high titers of natural (not induced by vaccine or passive administration) antibodies against onconeural antigens expressed on neurones and certain malignant cells. The antibodies are apparently induced by tumor growth and have been associated with autoimmune neurological disorders and, in addition, with delayed tumor progression and prolonged survival (23–25). (*b*) Patients with American Joint Commission On Cancer stage III melanoma and natural antibodies against GM2 ganglioside studied at two different medical centers have an 80–90% 5-year survival, compared to the expected 40% rate (26, 27). (*c*) Patients with small cell lung cancer and natural antibodies against small cell lung cancer had prolonged survival, compared to antibody-negative patients (28). (*d*) Patients with Dukes' C colon cancer treated with mAb 17-1A in the adjuvant setting had a significantly prolonged disease-free and overall survival, compared to randomized controls (29). (*e*) Antibody responses induced by vaccines in the adjuvant setting have been correlated with subsequent prolonged disease-free and overall survival (26, 27, 30–33).

Given the potential clinical importance of a variety of cell surface antigens, including ganglioside GD2, as targets for mAbs and cancer vaccines inducing an antibody response, we have identified a suitable syngeneic mouse model to address some of the variables associated with antibody-mediated protection from and therapy of cancer. EL4 is a lymphoma syngeneic in C57BL/6 mice that we have previously reported to express GD2 (34). It is a unique model, in that GD2 is also a human tumor antigen, against which there is not only a clinically active mAb but also a consistently immunogenic conjugate vaccine, GD2-KLH plus QS21. We demonstrate here that passively administered and vaccine-induced antibodies are able to prevent establishment of subsequently administered EL4 challenge and to eliminate EL4 micrometastases when administered after EL4 challenge, and we define some of the parameters of this protection.

MATERIALS AND METHODS

mAbs and Vaccine

The origins of mAb 3F8 (IgG3) against GD2 (35), mAb 696 (IgM) against GM2 (36), mAb 013 against a primitive human neuroectodermal bone tumor (37), and mAb IR3 against Tn antigen (38) have been described. Neither O13

Received 1/12/98; accepted 4/28/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by NIH Grants PO1 CA 33049 (to P. O. L. and G. R.) and RO1 CA 61017 (N-K. V. C.). P. O. L. is a paid consultant to and has share options in Progenics Pharmaceuticals, Inc., who has licensed the GD2-KLH vaccine from Memorial Sloan-Kettering Cancer Center. In exchange for the rights conveyed under the licensing agreement, Memorial Sloan-Kettering Cancer Center received shares in Progenics Pharmaceuticals, Inc., in lieu of a licensing fee.

[2] To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Fax: (212) 794-4352.

[3] The abbreviations used are: mAb, monoclonal antibody; KLH, keyhole limpet hemocyanin; CDC, complement-dependent cytotoxicity.

[4] The designations GM2, GD2, and GD3 are used in accordance with the abbreviated ganglioside nomenclature of Svennerholm (48).

Table 1 *Experiment 1: liver metastases after i.v. challenge with EL4 (lymphoma incubated with mAbs 3F8 (against GD2) and 696 (against GM2)*[a]

mAb	No. of mice	No. of tumors in liver	Liver mass (g)
PBS (control)	8	57.8 ± 67.2	2.59 ± 1.00
mAb 696	5	94 ± 100	2.52 ± 1.21
mAb 3F8	5	0	1.23 ± 0.04[b]
mAb 696 + mAb 3F8	5	0	1.20 ± 0.14[b]

[a] After incubation with 100 μg/ml 3F8 and 50 μg/ml 696 for 1 h, 3×10^4 EL-4 cells per mouse were injected (i.v.) into C57BL/6 mice. Thirty-four days after challenge, mice were sacrificed, and the livers were evaluated. Results are expressed as mean ± SD.
[b] $P < 0.01$, compared with PBS control group.

nor IE3 reacts with EL4. Immunological Adjuvant QS-21, a purified saponin fraction (39), was obtained from Aquilla Biopharmaceuticals Inc. (Worcester, MA). GD2 and GD2 conjugated to KLH were provided by Progenics Pharmaceuticals Inc. (Tarrytown, NY). Conjugation of GD2 to KLH was achieved by conversion of the GD2 ceramide double bond to aldehyde by ozonolysis and attachment to KLH by reductive amination in the presence of cyanoborohydride, as described previously for GD3 (40). Each GD2-KLH vaccine contained 10 μg of GD2 conjugated to 60 μg of KLH, plus 10 μg QS-21. Vaccines were administered s.c. three times at 1-week intervals, except in the final experiment, when they were administered at 4-day intervals.

Mice and Cell Lines

C57BL/6 mice (6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The EL4 cell line was established from lymphoma induced in a C57BL/6 mouse by 9,10-dimethyl-1,2-benzanthracene. It has recently been shown to express GD2 ganglioside (34). EL4 was maintained in 10% FCS-RPMI. For tumor cell challenges, EL4 cells were washed three times in PBS, and 3×10^4 cells (in the final experiment, 5×10^3 cells) were injected i.v. into the tail vein. At the indicated time points, mice were sacrificed, and livers were removed, weighed, and fixed in 10% formalin. Metastases were also frequently present in lymph nodes and other sites (although rarely in the lungs), but hepatic metastases were easiest to quantitate. Hepatic metastases were detected as white nodules on the liver surface.

Serological Assays

ELISA. ELISAs were performed as described previously (41). GD2 or GM2 in ethanol was coated on ELISA plates at 0.1 μg/well. A series of antiserum dilutions were incubated with the coated ganglioside for 1 h. Secondary antibodies were alkaline phosphatase-conjugated goat antimouse IgG or IgM at a dilution of 1:200 (Southern Biotechnology Associates, Inc., Birmingham, AL). ELISA titer is defined as the highest dilution yielding an absorbance of 0.1 or greater over that of normal control mouse sera. mAbs 3F8 and 696 were used as positive controls in each assay.

Flow Cytometry. EL4 cells (3×10^5) were incubated with 40 μl of 1:30 diluted antiserum or 1:2 diluted mAb supernatant for 30 min on ice. After washing with 3% FCS in PBS, the cells were incubated with 20 μl of 1:15 diluted FITC-labeled goat antimouse IgG or IgM (Southern Biotechnology Associates, Inc.). The positive population of the stained cells was quantitated by flow cytometry (EPICS-Profile II; Coulter Co., Hialeah, FL), as described previously (41).

CDC. In 100 μl of 5% FCS in RPMI, 2×10^5 EL4 cells were incubated with 10 μl of 1:10 mouse antiserum or 10 μg/ml mAb for 10 min. Thirty μl of complement (guinea pig; Sigma Chemical Co.) were added and incubated at 37°C for 4 h. Thirty μl of 0.4% trypan blue were added, and after 3 min, dead and viable cells were counted (41).

Statistical Methods

Experimental groups were compared to controls for number of hepatic metastases, survival, or antibody titers using the Mann-Whitney two-sample *t* test (42).

RESULTS

Having previously shown that mAb 3F8 was able to bind to EL4 and induce potent CDC and antibody-dependent cell-mediated cytotoxicity (3, 13, 35), we performed a series of experiments progressively testing the ability of passively administered and then actively induced antibodies against GD2 to eradicate hepatic micrometastases (experiments 1 and 2) and to prolong survival (experiments 3–7).

Effect of mAb Administration on Hepatic Metastases (Experiments 1 and 2). In experiment 1, we mixed 3F8 or negative control mAb 696 with the EL4 lymphoma cells prior to challenge to confirm *in vivo* impact of antibody binding. EL4 cells were incubated for 1 h with PBS, mAb 696 (against GM2, which is minimally expressed on EL4), mAb 3F8, or mAbs 696 and 3F8 prior to i.v. challenge. All mice were sacrificed on day 34, hepatic metastases were counted, and livers were weighed (Table 1). Only EL4 preincubation with 3F8 ± 696 eliminated metastases. In experiment 2, mice were injected i.v. with PBS, negative control antibody IE3 (100 μg), or one of three doses of 3F8 (50, 100, or 250 μg) 2 h before i.v. challenge with untreated EL4 cells. Mice were sacrificed at day 30. Administration of all three doses of 3F8 eliminated metastases in most mice (Table 2).

Effect of mAb Administration or Vaccination on Survival (Experiments 3–6). In experiment 3, two groups of six mice received a single i.v. injection of 200 μg of 3F8 1 day before or 2 days after EL4 i.v. challenge. Three additional groups of six mice were vaccinated three times (on days −21, −14, and −7) prior to EL4 challenge. They were vaccinated with PBS, 10 μg of GD2 mixed with 60 μg of KLH plus QS21 (negative controls), or 10 μg of GD2 conjugated to 60 μg of KLH plus QS21. Mice receiving the conjugate vaccine survived significantly longer than did the control mice ($P < 0.008$), and one mouse was sacrificed on day 100 with no evidence of tumor. Five of six mice receiving 3F8 1 day before challenge and five of six mice receiving 3F8 2 days after challenge also remained tumor free (Fig. 1, *Experiment 3*). All negative control mice died by day 28.

Experiments 4 and 5 focused on treatment with mAb. In experiment 4, groups of four or five mice received PBS or 3F8 2 days or 4 days after EL4 challenge i.v. All 3F8-treated mice survived longer than did control mice ($P < 0.004$), and three mice in the 3F8 groups remained tumor free (Fig. 1, *Experiment 4*). Experiment 5 compared treatment with PBS or mAb O13 (negative controls) and treatment with 50 or 200 μg of 3F8, all administered 2 days after EL4 challenge i.v. (Fig. 1, *Experiment 5*). Once again, all 3F8-treated mice survived longer than did any control mouse ($P < 0.004$), and most mice (8 of 12) treated with either dose of 3F8 remained tumor free.

Experiment 6 again compared immunization prior to tumor challenge with mAb treatment at various intervals after challenge. All vaccinated mice again survived longer than did any control mouse ($P < 0.004$), and four of six mice remained disease free (Fig. 1, *Experiment 6b*). Most mice receiving 70 μg of 3F8 2 or 4 days after challenge remained disease free. However, the same dose 7 or 10 days after challenge had no significant effect (Fig. 1, *Experiment 6a*). Experiment 6 was a single experiment but is presented in two panels for greater clarity. Once again, the relevant negative control treatments (mAb R24 against GD3, which is not expressed on EL4, and

Table 2 *Experiment 2: liver metastases after i.v. injection of mAbs followed by i.v. EL4 challenge*[a]

Treatment	No. of mice	No. tumors in liver	Liver mass (g)
PBS (control)	7	29.2 ± 14.8	1.90 ± 0.47
mAb IE3 (100 μg/mouse)	9	17.6 ± 15.9	1.95 ± 0.72
mAb 3F8 (50 μg/mouse)	6	0[b]	1.03 ± 0.13
mAb 3F8 (100 μg/mouse)	6	4.3 ± 7.0[c]	1.17 ± 0.41
mAb 3F8 (250 μg/mouse)	6	0[d]	0.90 ± 0.16

[a] Challenge was with 3×10^4 EL-4 cells 2 h after mAb injection. The mice were sacrificed 30 days after challenge, and the livers were evaluated. Results are expressed as mean ± SE.
[b] $P < 0.01$, compared with PBS control group.
[c] $P < 0.02$, compared with PBS control group.
[d] $P < 0.001$, compared with PBS control group.

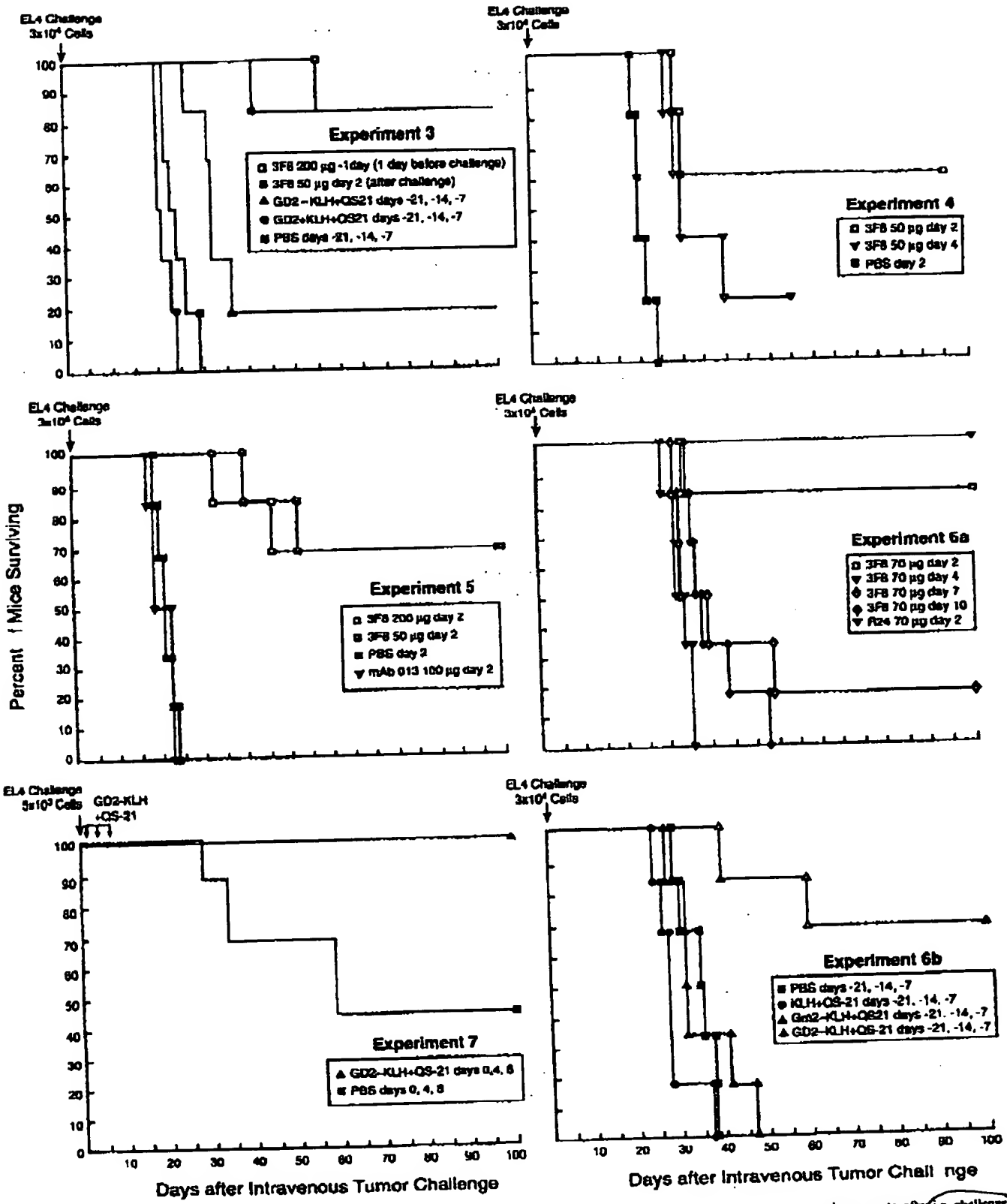

Fig. 1. Survival of groups of four to six mice treated in five separate experiments with 3F8 mAb, GD2-KLH plus QS-21 vaccine, and various control treatments, after i.v. challenge with syngeneic EL4 lymphoma cells. 3F8 mAb against GD2 administered prior to challenge or 1–4 days after challenge and GM2-KLH plus QS-21 vaccination prior to challenge or starting immediately after challenge were both protective.

Table 3 *Antibody reactivity in sera of mice treated with GD2-KLH vaccine or mAb 3F8*[a]

Treatment	No. of mice	Reciprocal ELISA titer		Flow cytometry (% positive cells)		CDC (% dead cells)
		IgM	IgG	IgM	IgG	
Experiment 3						
PBS (100 μl)	6	0/0	0/0	3.1–3.3/3.2	3.1–3.4/3.2	10–10/10
GD2 + KLH + QS21 (10 μg + 60 μg + 10 μg)	6	0–80/40	0	5.0–8.9/6.5	4.2–5.5/4.5	Not tested
GD2-KLH + QS21	6	40–640/80	80–2,560/640	18–88/57	22–93.6/60.5	20–60/40
3F8 (250 μg), 1 day before challenge	6	0/0	2,560–5,120/5,120	Not tested	99–99.3/99	90–95/95
Experiment 6						
PBS (100 μl)	6	0/0	0/0	1.5–2.9/2.0	1.4–2.6/1.7	10–15/10
KLH + QS21 (60 μg + 10 μg)	6	0/0	0/0	1.3–8.1/3.6	1.3–2.2/2.0	10–15/10
GD2-KLH + QS21	6	160–640/320	180–14,580/1,620	57–99/95	13–99/89	40–80/60
3F8 (250 μg)	6	0/0	1,620–4,860/3,240	Not tested	98–100/99	85–95/95

[a] Mice were bled 7 days after the third immunization with GD2 vaccine or 4–5 days after mAb 3F8 injection. Results are expressed as range/median.

vaccination with KLH plus QS21, GM2-KLH plus QS21, and PBS, which do not induce anti-GD2 antibodies) had no effect.

Correlation between Serum Antibody Titer and Survival. Serum anti-GD2 antibody titers immediately after 3F8 administration were not tested, but they ranged between 1:1620 and 1:4860 (median, 1:4860) 3–5 days later, except in experiment 4, in which they were between 1:180 and 1:4860 (median, 1:540). Vaccine-induced antibody titers ranged between 1:640 and 1:1620 for IgG and 1:80 and 1:1620 for IgM (Table 3). Comparable antibody titers by ELISA resulted in comparable reactivity by flow cytometry and complement-mediated cytotoxicity, whether due to 3F8 or vaccine administration. In both cases, protection from subsequent tumor challenge resulted. A correlation between antibody titer and *in vivo* protection is suggested by these results. Administration of 3F8 resulted in higher serum titers against GD2 than vaccine administration in both experiments ($P < 0.004$ for CDC) and greater protection ($P < 0.008$ for experiment 3). In experiment 4, in which 3F8 levels were lower than expected after 3F8 administration, survival was lower as well. Vaccine-induced antibody titers prior to challenge were higher in experiment 6 than in experiment 3, and protection was greater as well ($P < 0.025$).

Therapeutic Vaccination. Because 3F8 administration 7 or 10 days after EL4 challenge with 3×10^4 resulted in minimal protection, this suggested that vaccination after challenge, which was normally performed at weekly intervals and required 14–21 days for antibody induction, would be ineffectual. Consequently, we performed one final experiment aimed at testing the ability of vaccinations started after tumor challenge to prolong survival. In experiment 7, the number of EL4 cells per challenge was decreased from 3×10^4 to 5×10^3 cells, and the vaccines were administered on days 0, 3, and 7, beginning immediately after the challenge. Median IgM and IgG antibody titers on days 13 and 18 were both 1:320. Protection was again seen (Fig. 1, *Experiment 7*), although the difference was not statistically significant ($P = 0.15$).

DISCUSSION

The mechanism of antibody effect against bacteria is predominantly complement mediated inflammation and cytotoxicity (CDC; Ref. 43). Although other effector mechanisms have been suggested for GD2 antibody, such as inhibition of tumor cell substratum or extracellular matrix interactions (22), activation of immune effector mechanisms remains the most likely explanation. 3F8, the anti-GD2 mAb used here, is an IgG3 antibody that is particularly potent at inducing complement-mediated inflammation/cytotoxicity and antibody-dependent cell-mediated cytotoxicity. We have previously demonstrated, in melanoma patients, that natural or vaccine-induced IgM antibodies against GM2 ganglioside correlated with improved disease-free and overall survival (26, 44) and that a GM2-KLH plus QS21 vaccine induced IgM and IgG antibodies in melanoma patients, which were both able to mediate CDC (45). Fortuitously, the IgG subclasses were IgG1 and IgG3 (44–46), the two human subclasses best able to mediate CDC. The same applies to the murine model we describe here. IgM and IgG antibodies were induced in all vaccinated mice, these antibodies and administered 3F8 mAbs were able to mediate potent CDC, and antibody titers correlated with survival and inversely with the number of hepatic metastases. Although mAbs administered up until 4 days after challenge were able to completely prevent tumor growth in most mice, by 7–10 days after challenge, 3F8 administration had little effect. This strongly suggests that treatment with mAbs or vaccines inducing antibodies must be restricted to the adjuvant setting, where the targets are circulating tumor cells and micrometastases, and it may explain why mAb treatment trials in patients with measurable tumor burdens have not been more successful.

Passively administered and vaccine-induced antibodies were both able to protect against growth of micrometastases. There are advantages and disadvantages to each approach. Therapy in the adjuvant setting may require repeated treatments to maintain antibody titers over a prolonged period to overcome the issue of tumor cell dormancy and sanctuary sites. Except in immunosuppressed patients, this excludes murine mAbs, which would be eliminated within weeks by human antimouse antibodies. Chimeric, humanized, or human mAbs would overcome this issue but would be subject to elimination by anti-idiotypic antibodies. On the other hand, in the absence of human antimouse antibodies or anti-idiotypic antibodies, higher serum antibody levels than could be induced by vaccination are assured after mAb administration, and such antibodies have been or could be produced against most antigens. Vaccines against most defined tumor antigens are more practical to produce and administer because they can be administered s.c. and at longer intervals. Phase III trials with GM2-KLH and sialyl Tn-KLH vaccines that consistently induce moderate titers of antibodies against these antigens are currently ongoing in the adjuvant setting in patients with melanoma and breast cancer (33, 45). Because the antibody response seems to be polyclonal, antibody inactivation by anti-idiotypic antibodies has not been a problem and specific antibody levels have been maintained against GM2 by immunizations at 3- or 4-month intervals for over 2 years (45). However, even the most potent conjugate vaccines have not been able to induce consistent antibody responses against all antigens, and the titers are never as high as can be achieved with mAb administration. The results obtained here, demonstrating the ability of either approach to protect against tumor challenge and to eliminate micrometastases, in the absence of any detectable toxicity, argue strongly in favor of the careful use of either approach or the combination.

GM2-KLH and GD2-KLH have both proven consistently immunogenic and safe in melanoma patients, whereas GD3 (the major melanoma ganglioside)-KLH has not proven so immunogenic (reviewed in Ref. 47). Adjuvant therapy of melanoma might optimally include a bivalent conjugate vaccine (GM2-KLH plus GD2-KLH), a humanized anti-GD3 mAb, or a combination of bivalent vaccine plus mAb.

Vaccines against infectious diseases do not prevent infection; they limit its spread from its point of contact. Postcontact boosts in antibody titers, even in protected hosts, attest to active infection at the contact site. This is most striking when time has elapsed because the original infection and antibody titers have fallen to low levels but rise to protective levels within 4–7 days, preventing symptomatic infection. In patients with cancer, we see the adjuvant setting (after removal of the primary cancer or positive lymph nodes) as being quite similar to the picture in patients being reexposed to infectious diseases. The primary targets in both cases are circulating pathogens and microscopic spread, and in the case of infectious diseases, antibodies are the primary method of protection. We demonstrate here, with passively administered mAbs and vaccine-induced antibodies against the defined cancer antigen GD2 ganglioside, that antibodies can also protect mice against circulating syngeneic tumor cells and micrometastases. If antibodies of sufficient titer and potency to eliminate circulating cancer cells and micrometastases could be maintained in cancer patients as well, even metastatic cancer would have quite a different implication. With continuing showers of metastases no longer possible, aggressive treatment of primary and metastatic sites might result in long-term control.

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